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13. ABSTRACT (Maximum 200 Words)
Signaling of insulin-like growth factor-I (IGF-I) through the type I insulin-like growth factor receptor (IGF-IR) has been shown to regulate breast cancer cell proliferation, survival and metastasis in vitro. Recent evidence indicates insulin receptor substrate-1 and -2 (IRS-1 and IRS-2), the primary signaling molecules utilized by the IGF-IR, may mediate distinct IGF-I effects. To investigate the specific functional roles of the IRS species in mediating IGF action, we utilized the T47D-Y and T47D-YA breast cancer cell lines, which lack both IRS-1 and IRS-2 expression yet express a functional IGF-IR, to generate cell lines that independently express IRS-1 or IRS-2. T47D-Y and T47D-YA breast cancer cells were stably transfected with either human IRS-1 or IRS-2 cDNA, screened for IRS expression and activation by immunoblotting, and analyzed for IGF responsiveness in proliferation and motility assays. T47D-YA/IRS-1 cell clones exhibited a proliferative response to IGF-I stimulation, whereas T47D-YA/IRS-2 cell clones did not proliferate in response to IGF-I. However, both T47D-YA/IRS-1 and T47D-YA/IRS-2 cells exhibited IGF-I stimulation of cell motility. Therefore, data from this study-in-progress suggests the IRS species may play specific functional roles in mediating certain aspects of IGF action (proliferation), while potentially playing redundant roles for other IGF effects (motility).

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Cell signaling, insulin-like growth factor, insulin receptor substrate, cancer cell proliferation and migration

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INTRODUCTION: The insulin-like growth factor (IGF) system is a key regulatory pathway in breast cancer cell proliferation, metastasis, and protection from apoptosis. IGF exerts these biological effects through activation of the type I insulin-like growth factor receptor (IGF-IR), resulting in a rapid phosphorylation of intracellular docking proteins, such as insulin receptor substrate-1 and -2 (IRS-1 and IRS-2)(1,2). While various signaling intermediates of the IGF-IR have been identified, our understanding of the specific functional roles of these molecules with respect to IGF-I mediated effects is limited. However, recent evidence indicates IRS-1 and IRS-2, two of the primary signaling intermediates utilized by the IGF-IR, may mediate distinct aspects of IGF-I signaling. Breast cancer cells where IRS-1 is the primary signaling molecule activated upon IGF-I stimulation exhibit enhanced cell proliferation and protection from apoptosis(3). In contrast, breast cancer cells that predominantly signal through IRS-2 exhibit increased metastatic behavior(4). Currently, breast cancer cell lines do not exist that allow the differentiation between IRS-1 and IRS-2 mediated effects. In this study, the T47D-Y and T47D-YA cell lines were used to develop cell lines that would allow this differentiation. The ultimate goal of the present study is to identify differential roles of the IRS proteins in mediating IGF-I effects in breast cancer cells and to elucidate the different signaling pathways utilized to account for the differences in effect.

BODY:

Specific Aim #1: *Generate T47D-Y and T47D-YA cell lines that independently express IRS-1 or IRS-2.*
Human IRS-1 and IRS-2 cDNA were stably transfected into T47D-Y breast cancer cells, followed by selection with 500 ug/ml Geneticin G418 in culture medium. As T47D-YA cells were generated under G418 selection criteria(5), IRS-1 and IRS-2 were introduced into these cells by cotransfection of the IRS construct and a vector encoding hygromycin resistance, followed by selection with 200 ug/ml hygromycin in culture medium. Although a deviation from the original statement of work, the overall goal of generating T47D-YA cells that differentially express IRS-1 and IRS-2 was accomplished. Stably transfected cell clones were isolated that express and activate each of the IRS proteins of interest (Figure 1 and Figure 2). These results establish cell lines that can be used to study the specific functional roles of IRS-1 and IRS-2 in mediating IGF-I action in breast cancer cells.

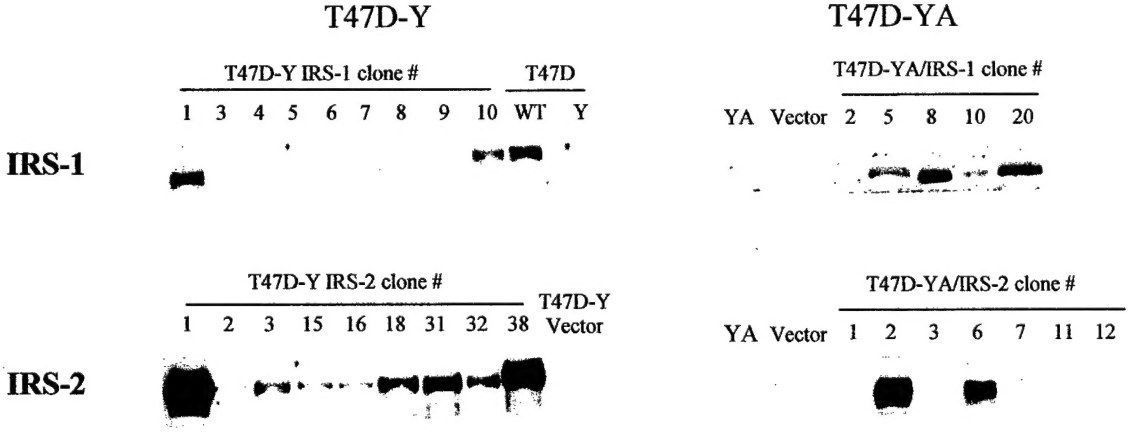

Figure 1 Generation of T47D-Y/IRS-1 and T47D-YA/IRS-1 stable cell lines that express IRS-1 protein and T47D-Y/IRS-2 and T47D-YA/IRS-2 stable cell lines that express IRS-2 protein.
Human IRS-1 and IRS-2 cDNAs were generously supplied by Dr. Adrian Lee (Baylor College of Medicine). T47D-Y and T47D-YA cells were generously supplied by Dr. Kathryn B. Horwitz (University of Colorado Health Sciences Center). pcDNA 3.1 (-) IRS-1 or IRS-2 cDNA was stably transfected into T47D-Y using Effectene transfection reagent, followed by selection with 500 ug/ml Geneticin G418 in culture medium. As T47D-YA cells were generated under G418 selection criteria, IRS-1 or IRS-2 were introduced into these cells by cotransfection of the IRS construct and a vector encoding hygromycin resistance, followed by selection with 200 ug/ml hygromycin in culture medium. Stable clones were allowed to form and then

individually transferred into 24 well plates and expanded. Clones were screened for IRS-1 or IRS-2 expression by immunoblotting with an anti-IRS-1 or anti-IRS-2 antibody, both from Upstate.

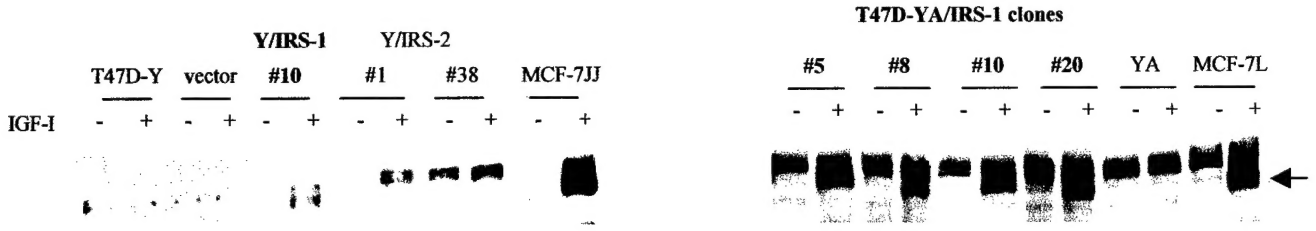

Figure 2 The IGF-I system is functional in the T47D-Y and T47D-YA cells stably transfected with IRS-1 and IRS-2. T47D-Y/IRS-1, T47D-Y/IRS-2, and T47D-YA/IRS-1 cell clones were examined for activation of their respective IRS protein. Activation of T47D-YA/IRS-2 clones is currently under investigation. Cells were plated at a density of 1x106 cells/dish, grown to 70% confluence, serum-starved overnight, and treated for 10 minutes with or without 5nM IGF-I. 40ug of total cell lysates were run on an 8% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with an antibody directed towards phosphorylated tyrosine residues.

Specific Aim #2: *Examine the phenotypic effects of IRS-1 and IRS-2 protein expression on IGF stimulated cell proliferation, migration, and protection from apoptosis.*

Cell proliferation was measured in T47D-YA/IRS-1 and T47D-YA/IRS-2 cell clones using the 3-[4,5-dimethylthiazol 2-yl] 2,5-diphenyltetrazolium bromide (MTT) growth assay as previously described(6). Whereas the T47D-YA cells do not proliferate in response to IGF-I stimulation, four of the five T47D-YA/IRS-1 cell clones did exhibit an IGF-I stimulated cell proliferation (Figure 3). These data indicate that expression of IRS-1 in cells lacking IRS-1 results in introduction of IGF-I mediated stimulation of cell proliferation. In contrast, two of the three T47D-YA cell clones expressing IRS-2 did not exhibit a mitogenic response to IGF-I in this assay (Figure 3). The IRS-2 clone that was found to respond to IGF-I with an increase in proliferation was found to have compensatory protein expression of IRS-1 (data not shown). Together, this data suggests that IRS-1 may play a specific functional role in mediating IGF-I stimulated cell proliferation.

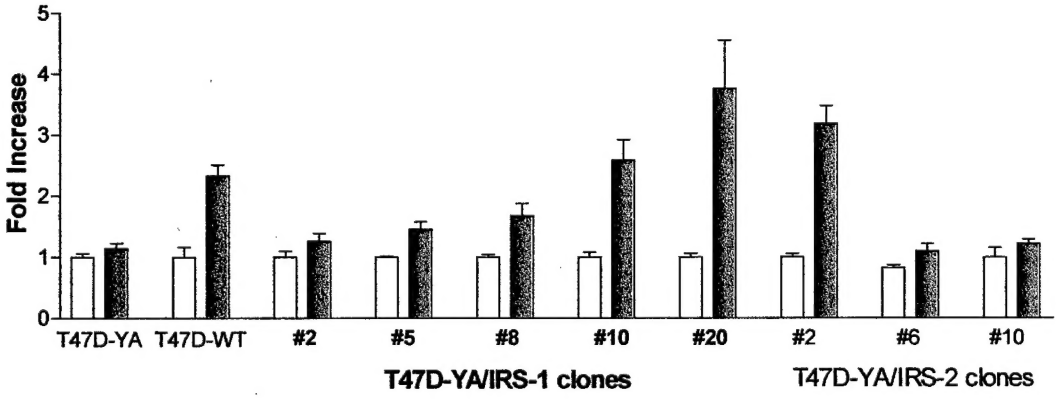

Figure 3 Introduction of IRS-1 but not IRS-2 into T47D-YA cells restores the mitogenic response to IGF-I seen in T47D-WT cells. 10,000 cells were plated in each well of a 24 well plate in triplicate, treated with or without IGF-I, incubated at 37°C for six days, and the MTT assay performed. Results are presented as fold increase of absorbance of IGF-I treated samples over absorbance of non-treated samples. Empty bars represent cells untreated control cells and dark bars represent cells treated with IGF-I.

While the MTT assay was effective in determining the mitogenic effects of IGF-I in T47D-YA cells expressing IRS-1 and IRS-2, an elevated basal cell growth of the T47D-Y cell clones prohibited a similar study of IGF-I mediated proliferation in the T47D-Y cells expressing IRS-1 and IRS-2 using this assay. Thus, measurement of IGF stimulation of cells into the S phase of the cell cycle by flow cytometry is currently being investigated as an alternative method to determine the mitogenic effects of IGF-I in T47D-Y cells expressing IRS-1 or IRS-2.

The motility response of the IRS-1 and IRS-2 expressing cell clones to IGF-I was assessed by both the Boyden chamber assay and gold particle motility assay, two methods commonly used to study cell motility. T47D-Y cells expressing IRS-2 exhibited an IGF-I stimulated increase in cell motility in both assays (Figure 4A,B). In contrast, T47D-Y cells expressing IRS-1 had no enhancement in motility upon IGF-I stimulation in the gold particle assay (Figure 4B). This data suggests IRS-2 may play a specific role in mediating IGF-I stimulation of cell motility. However, T47D-YA cells expressing either IRS-1 or IRS-2 exhibited IGF-I stimulation of motility in the Boyden Chamber assay (Figure 4C). Together, these results suggest both IRS-1 and IRS-2 have the potential to mediate IGF-I stimulation of cell motility. Additional studies are needed to investigate the differential response of the T47D-Y and T47D-YA cells stably transfected with IRS-1.

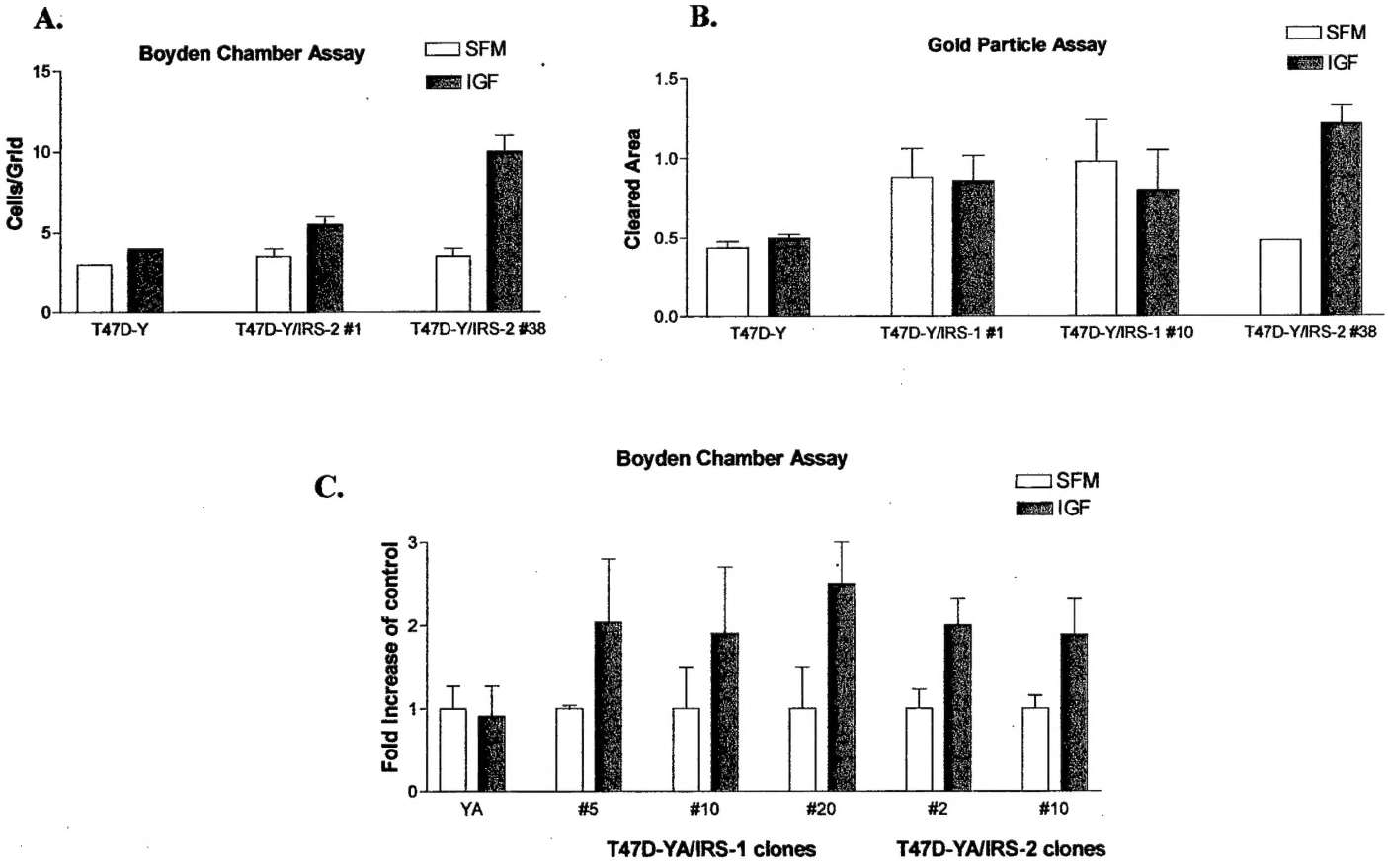

Figure 4 IRS-1 and IRS-2 may both mediate IGF-I stimulation of cell motility *A,C.* The Boyden chamber assay measures cell motility using a Neuro Probe 10 well chemotaxis chamber. SFM +/- 5nm IGF-I was placed in the bottom of a Boyden chamber, a polycarbonate filter placed on top, and 0.3 mL of cell suspension ($5x10^5$ cells/ml) added to the top of wells. Cells

were incubated for 6 hours, upon which the cells that had migrated to the bottom of the filter were stained with HEMA3 (Fischer) and counted in five areas for each well using a light microscope. *B.* In the gold particle assay, coverslips were coated with a gold particle solution and 50,000 cells plated per coverslip. The cells were allowed to adhere, treatments of SFM +/- IGF-I applied, and the cells incubated at 37° C in humidified air with 5% CO_2 for 24 hours. Cells were then fixed with glutaraldehyde and coverslips mounted on slides. Coverslip images are captured using a brightfield microscope with a neutral density filter and the area on the coverslip cleared by cell movement is computed using Image Pro Plus Alias. The program generates a number representing the mean area cleared, which correlates to the motility of the cells.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of cell lines that can be used to study the specific functional roles of IRS-1 and IRS-2 in mediating IGF-I action in breast cancer cells
- Investigation of the roles of IRS-1 and IRS-2 in mediating IGF-I stimulated cell proliferation and motility

REPORTABLE OUTCOMES:

- Abstract: Expression of Insulin Receptor Substrate-2 (IRS-2) in T47D-Y Breast Cancer Cells Induces an IGF-I Mediated Cell Motility **S. Byron**, X. Zhang, D. Sachdev, D. Yee
  Gordon Research Conference: Insulin-Like Growth Factors in Physiology and Disease
  March 2003

CONCLUSIONS:

IGF action is an increasingly important focus for breast cancer research due to its regulation of breast cancer cell proliferation, metastasis, and protection from apoptosis. Continuation of this work will test the hypothesis that IRS-1 and IRS-2 mediate distinct aspects of this malignant phenotype and will further investigate whether different signaling pathways are utilized by these two molecules to mediate specific actions of IGF. Thus far, data from this study suggests the IRS species may play specific functional roles in mediating certain aspects of IGF action, while for other IGF effects the IRS molecules may play redundant roles.

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